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Evaluation of Two Simplified Methods for Genotyping Hepatitis C Virus

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A number of different approaches have been used for genotyping hepatitis C virus (HCV). Two simplified methods were evaluated, both of which used polymerase chain reaction (PCR) to amplify products from the 5′ non-coding region of HCV: non-isotopic restriction fragment length polymorphism (RFLP) analysis and type-specific PCR. Sixty-four viraemic patients suffering from chronic HCV infection were studied using these two techniques; 25/64 samples were further tested with a commercial serotyping ELISA based on synthetic NS4 antigen (Murex, U.K.). The results of the three typing methods were generally in agreement with each other. When only the predominant genotype identified by each method was analysed, the 3 methods had 100% agreement. RFLP did not detect any mixed infections and it was unsuccessful in 16/64 (25%) samples. Both type-specific PCR and serotyping ELISA detected mixed infections. However, serotyping ELISA did not give typeable results in 7/25 (28%) samples, whereas type-specific PCR gave typeable results in all 64 samples. Type-specific PCR detected more mixed infections than serotyping ELISA. Direct sequencing of four PCR products with indeterminate RFLP confirmed changes in restriction enzyme recognition sites. The sequences also confirmed the validity of the predominant genotype in cases of apparent mixed infections. It is possible that some of these cases were artefacts as a result of quasispecies. ***J. Med. Virol. 52:35–41, 1997.***

KEY WORDS: RFLP; type specific PCR; serotypes; mixed genotype HCV infection

INTRODUCTION

Identification of hepatitis C virus (HCV) as the major cause of parenterally transmitted non-A, non-B hepatitis [Choo et al., 1989] has resulted in subsequent cloning and sequencing of a number of HCV isolates from different geographical areas. On the basis of these complete or partial sequences homology analyses, distinct HCV genotypes have been described [Choo et al., 1991; Okamoto et al., 1992; Chan et al., 1992; Simmonds et al., 1993a]. Genotypic differences are most useful for the study of global HCV epidemiology. The importance of HCV genotypes is further emphasised by the association of specific genotype (type 1, particularly 1b) with severity and poor response to interferon therapy [Pozatto et al., 1991; Kanai et al., 1992; Nousbaum et al., 1995].

Nucleotide sequencing remains the most definitive method for identifying different genotypes of HCV [Chan et al., 1992; Simmonds et al., 1993a], but this is not possible for routine clinical studies. More practical methods include: direct polymerase chain reaction (PCR) of clinical samples using type-specific primers which amplify selectively different genotypes [Okamoto et al., 1992], the use of genotype-specific probes to hybridise with PCR products [Stuyver et al., 1993; Van Doorn et al., 1994], restriction fragment length polymorphism (RFLP) analysis of DNA amplified by PCR [Nakao et al., 1991; McOmish et al., 1994], or by serotyping methods using type-specific synthetic peptides [Simmonds et al., 1993b; Bhattacherjee et al., 1995; Dixit et al., 1995]. Although these methods are less complicated than cloning and sequencing, most methods are still too complicated or too costly to allow rou-

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tine diagnostic use. We evaluated 2 modified methods of genotyping HCV using non-isotopic RFLP analysis and type-specific PCR, both based on the amplification of the conserved 5′ non-coding region (5′ NCR). The consensus genotype nomenclature system proposed by Simmonds et al. [1994] was adopted in this study.

PATIENTS AND METHODS

Serum samples from 64 British patients from the Mersey region of England suffering from chronic HCV infection with proven viraemia were used in this study. Risk factors for the acquisition of hepatitis C included: haemophiliacs (n = 39), intravenous drug users (n = 16), haemodialysis patients (n = 2), post-transfusion patient (n = 1), health care worker (n = 1), and sporadic unknown causes (n = 5). All patients were positive for anti-HCV using third-generation ELISA (Murex, UK or Ortho, US) and viraemia was determined by PCR (Amplicor, Roche, Switzerland).

RFLP

This was a modification of the method described by McOmish et al. [1994]. Viral RNA was extracted from 50 μl of serum using standard technique of proteinase K digestion, phenol chloroform extraction, and ethanol precipitation [Sambrook et al., 1989]. The pelleted nucleic acid was resuspended in 50 μl of diethyl pyrocarbonate treated water. Reverse transcription was performed on 11.5 μl of the resuspended nucleic acid using 50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 250 μM of each dNTP, 1.5 μM universal external anti-sense primer HC2 (position −66 to −85 [Choo et al., 1991], 5′ TTTCGCRACCCAACRCTACT), 20 units RNAguard (Pharmacia, Sweden), and 200 units of Moloney Murine Reverse Transcriptase (GibcoBRL, Life Technologies Inc, US) in 20 μl at 37°C for 60 minutes. First round PCR was carried out on 10 μl of cDNA using 20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.4 μM each of universal external anti-sense primers HC2 and universal external sense primer HCl (position −260 to −241, 5′ GCCATGGCGTTAGTAYGAGT), and 0.6 units of Taq DNA polymerase (GibcoBRL, Life Technologies Inc, US) in 25 μl with oil overlay. The PCR reaction consisted of 35 cycles each with 1 minute at 94°C, 1 minute at 40°C, and 1 minute at 72°C using an automated thermocycler (Omnigene, Hybaid, UK). The first round product was diluted 20-fold in water and 5 μl was used in a second round reaction using the same reaction mix as the first round but replacing the primers with universal internal sense primer HC3 (position −243 to −224, 5′AGTGTCRTRCAGCCTCCAGG) and universal internal anti-sense primer HC4 (position −73 to −92, 5′ ACCCAACRCTACTMGGCTAG). The effective volume of the first round input to second round was 0.25 μl. A small volume was used to prevent non-specific bands carried over from the first. Other PCR conditions were the same as above. The resultant 171 base pair PCR product, which was shorter than that used by McOmish et al. [1994] but within the same stretch of nucleotide sequence, was purified using the Wizard PCR Prep Purification kit (Promega, US) according to the manufacturer's instructions. Purified product was resuspended in 50 μl of water. Fifteen microlitres of the purified PCR product was digested by either *Hinf*1/*Mva*1 in SuRE/Cut buffer H or *Hinf*1/*Scrf*1 in SuRE/Cut buffer B (Boerhinger-Mannheim, Germany) at 37°C for one hour. The digested fragments were separated by electrophoresis in a 6% Nusieve GTG agarose gel (Flowgen, US) stained with ethidium bromide (0.05% w/v). The separated fragments were visualised

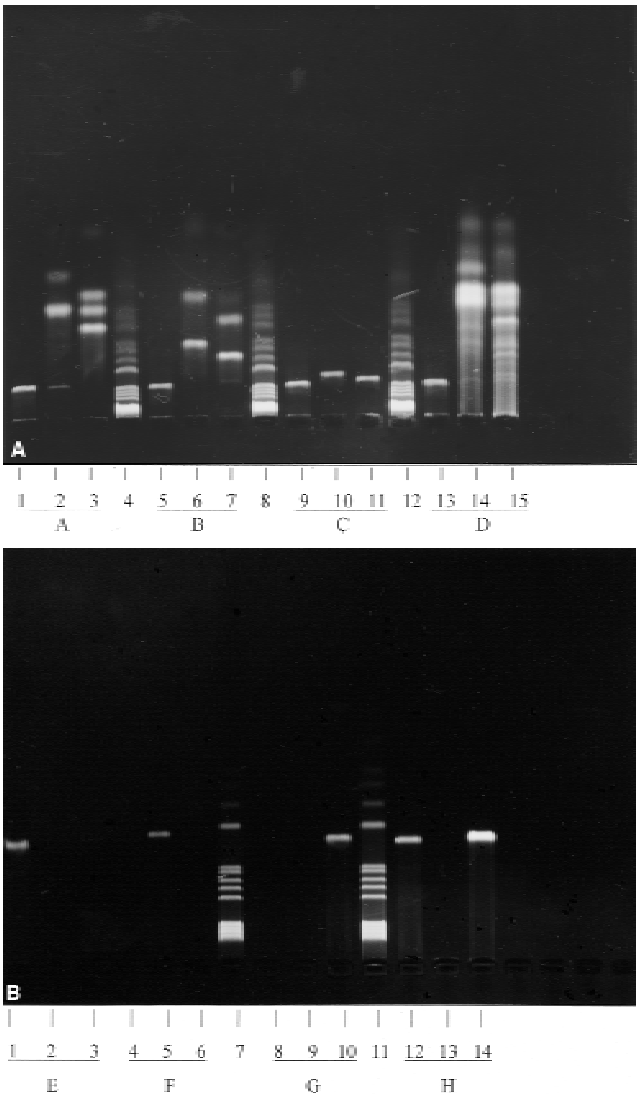

Fig. 1. **(A)** RFLP digestion patterns illustrated by samples A (genotype 1), B (genotype 3), C (genotype 2), and D (indeterminate). Uncleaved PCR products were run in lanes 1, 5, 9, and 13; *ScrfI*/*HinfI* digested PCR products were run in lanes 2, 6, 10, and 14; *MvaI*/*HinfI* digested PCR products were run in lanes 3, 7, 11, and 15. Lanes 4, 8, and 12 contain marker pBR322 Hae III digest. **(B)** Type-specific PCR illustrated by samples E (genotype 1), F (genotype 2), G (genotype 3), and H (mixed infection 3 > 1). Genotype 1 specific primers were run in lanes 1, 4, 8, and 12. Genotype 2 specific primers were run in lanes 2, 5, 9, and 13. Genotype 3 specific primers were run in lanes 3, 6, 10, and 14. Lanes 7 and 11 contain marker pBR 322 Hae III digest.

TABLE I. Predicted RFLP Digestion Patterns*

Restriction enzymes	Patterns	Genotype	Visible fragments (bp)
*Mva*1/*Hinf*1	A	1	63,50,41
	B	6	63,50,44
	C	3,4	98,56
	D	2,5	154
*Scrf*1/*Hinf*1	a	1,5	50,48,32
	b	1	57,50,32
	c	2	50,48,41
	d	2	139
	e	2	91,48
	f	3	82,41
	g	4	50,41,32
	h	6	50,48,35

*Based on McOmish et al., 1994.

by ultraviolet transillumination (Figure 1A). Digestion patterns were then compared with the expected patterns from known HCV genotypes (Table I) as deduced by McOmish et al. [1994] based on the analysis of 120 available sequences.

Type-Specific PCR

Type-specific PCR was described originally by Okamoto et al. [1992] which used type-specific primers to amplify the core region of HCV. We, however, designed novel type-specific primers for the three major genotypes 1, 2, and 3 and subtype 1b to amplify the 5′ NCR. RNA was extracted as before. Reverse transcription and the first round of PCR was identical to that described in RFLP. The second round was performed in microtitre plates using an Omnigene thermocycler (Hybaid, UK) with the universal internal sense primer HC3 and type-specific anti-sense primers (genotype 1: position −121 to −102, 5′ GGGGCACGCCCAAATCTCCA; genotype 2: position −131 to −112, 5′ CAAATGACCGGRCATAGAGT; genotype 3: position −124 to −105, 5′ GCACGCCCAAATTTCTGGGT; and subtype 1b: position −98 to −79, 5′ ACTACTCGGCTAGCAGTCTC). A separate reaction was carried out in individual microtitre wells for each genotype or subtype. Reaction conditions were optimised to 35 cycles of 94°C for 1 minute, 55.5°C for 1 minute, and 72°C for 1 minute. The PCR product from each well was separated by electrophoresis in a 3% agarose gel stained with ethidium bromide and visualised by ultraviolet transillumination for the diagnostic band which is 145 bp for type 1, 135 bp for type 2, 142 bp for type 3, and 168 bp for subtype 1b. The PCR products from each genotype or subtype reaction were run in different lanes (Fig. 1B). Accurate size discernment was therefore not important. In cases where there were PCR products for more than one genotype, the intensity of each band was scored and the genotypes were ranked according to band intensity.

Serotyping ELISA

Twenty-five samples that had sufficient serum left after RFLP and type-specific PCR were further tested with a commercial serotyping assay (Murex 1–6 assay, Murex, UK). This serotyping ELISA is based on the detection of type-specific antibodies against synthetic type-specific NS4 antigen. Briefly, a diluted serum sample was incubated in the presence of heterologous competing peptides, with microwells coated with serotype-specific antigens of HCV. During the first incubation any serotype-specific anti-HCV antibodies in the sample bound to the immobilised antigens. After washing to remove unbound material, the captured anti-HCV antibodies were incubated with peroxidase-conjugated monoclonal anti-human IgG. During the second incubation an antigen—human antibody—anti-human antibody/enzyme complex was formed in those microwells which contained samples with antibodies to a specific serotype of HCV. After removal of excess conjugate, bound enzyme was detected by the addition of solution containing 3,3′, 5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide. A purple colour developed in the wells which contained anti-HCV reactive samples. The enzyme reaction was stopped with sulphuric acid to give an orange colour, which was then read photometrically. The amount of conjugate bound and hence colour in the wells was directly proportional to the concentration of specific antibody in the sample. The manufacturer's instructions were followed in assigning of the serotypes. In cases of mixed infections, the serotype with the highest optical density reading was taken as the predominant type. Previous studies have shown a good correlation between serotypes and genotypes [Simmonds et al., 1993b; Bhattacherjee et al., 1995], and therefore serotypic results were taken as interchangeable with genotypic results in this study.

Sequencing of the PCR Products

Four samples with evidence of mixed infections and/or indeterminate RFLPs (n = 4) were further investigated by direct sequencing of the PCR product. PCR products were purified using the Wizard PCR prep purification kit (Promega, US) and the purified PCR products were sequenced bidirectionally using the universal forward and reverse primers HC3 and HC4 (373A DNA Sequencer, Applied Biosystems, UK). Between three and five reactions were purified and sequenced from each sample to establish a consensus sequence. The sequences were then aligned with the prototypes and pairwise sequence similarity was calculated using the computer software Megalign (DNAstar, Madison, WI).

RESULTS

RFLP

All samples produced a visible product after nested PCR with the universal primers on ethidium bromide stained agarose gel electrophoresis. However, only 55/64 (86%) produced sufficient PCR products for further digestion with restriction enzymes. The 9 samples (14%) with insufficient PCR products could not be tested with RFLP even after pooling of multiple reactions. With the remaining samples, RFLP identified

TABLE II. Samples Showing Evidence of Mixed Infection by One or More Methods

RFLP	Type-specific PCR	ELISA
1	1 > 3	Untypeable
1	1 > 2	1
1	1 > 3	1 > 6 > 2
1	1 > 2	1
1	1 > 2	Untypeable
2	2 > 1	2 > 1 > 4
2	2 > 1	2
3	3	3 > 2
3	3 > 1	Untypeable
Indeterminate	1 > 2 = 3	Untypeable
Indeterminate	1 > 3 > 2	1
Indeterminate	1 > 3 > 2	1

33/55 patients (60%) as genotype 1, 4/55 (7%) as genotype 2, and 11/55 (20%) as genotype 3 (Figure 1A). Seven samples (13%) had restriction digestion patterns that did not fall into any of the predicted patterns and their genotypes were therefore indeterminate. Overall, this approach was successful in assigning genotypes to 48/64 (75%) samples. Improvement in PCR efficiency should improve the success rate as insufficient PCR product, not the RFLP method itself, was the problem in 9/64 (14%) cases.

Type-Specific PCR

Type-specific PCR was successful with all 64 samples. Mixed infections were evident in 11 samples (17%) in which there were bands associated with more than 1 genotype. If the predominant band only was used in analysis, type-specific PCR identified 44/64 (67%) as type 1, of which 8 had evidence of mixed infection; 4/64 (6%) were identified as type 2, of which 2 had evidence of mixed infection; 16/64 (25%) were identified as type 3, of which 1 had evidence of mixed infection. Of the 44 samples with genotype 1 infection, 27 (61%) were also positive with the subtype 1b primer. None of the non–type 1 genotypes reacted with the type 1b–specific primers.

RFLP vs. Type-Specific PCR

Of the 48 samples that had a typeable result by both RFLP and type-specific PCR, 40/48 (83%) samples had concordant results: 28 were genotype 1, two were genotype 2, and 10 were genotype 3. The 9 samples with insufficient PCR product for RFLP analysis were all typeable with type-specific PCR: 6 were genotype 1 and 3 were genotype 3. Of the 7 samples which gave an indeterminate pattern with RFLP, 2 were genotype 1, two were genotype 3, and 3 had triple mixed infection with genotypes 1, 2, and 3 (genotype 1 was predominant in all). The results for the remaining 8 samples which showed disagreement between the 2 methods were all mixed infections as identified by type-specific PCR (Table II). However, if only the predominant band from the mixed infections was considered, then the results of the typeable samples were in complete agreement with type-specific PCR.

Serotyping ELISA

Serotyping ELISA was carried out on 25 samples which had sufficient serum left after RFLP and type-specific PCR. Eleven of these 25 were samples that had complete RFLP and type-specific PCR agreement. Serotyping ELISA also agreed in 7/11 (64%); 3 samples were untypable by ELISA; 1 sample which both RFLP and type-specific PCR identified as type 3, serotyping ELISA identified as mixed infection of type 2 and type 3 (type 3 predominant). Of the 3 samples indeterminate with RFLP but typable with type-specific PCR, 1 was serotype 1 and 2 were serotype 3: serotyping ELISA agreed with type-specific PCR on all. The remaining 11 samples all had mixed infections as identified by type-specific PCR: serotyping ELISA identified 2 of these 11 as mixed infections (Table II). If only the predominant type was considered, the typeable results of serotyping ELISA were in complete agreement with both RFLP and type-specific PCR.

Risk Factors

Combining the results of the 3 methods and using the predominant genotypes only, patients were categorised according to their risk factors. Of the 39 haemophiliacs, 30 (77%) were genotype 1, two (5%) were genotype 2, and 7 (18%) were genotype 3. Of the 16 intravenous drug users, 10 (63%) were genotype 1, one (6%) was genotype 2, and 5 (31%) were genotype 3. The single post-transfusion patient had genotype 2 infection. The patient whose only risk factor was being a health care worker had genotype 3 infection. Of the 2 patients on haemodialysis, 1 had genotype 1 and the other had genotype 3 infection. In 5 patients where the route of infection was not known, 3 were genotype 1 and 2 were genotype 3. Comparing the 2 main groups of patients—haemophiliacs and intravenous drug users—there was no statistically significant difference in their genotype distribution ($\chi^2 = 1.27$ and $P = .529$). Assuming that the mixed infections as identified by type-specific PCR were correct, then 6/39 (15%) haemophiliacs, 3/16 (18%) intravenous drug users, and 2/2 (100%) haemodialysis patients had mixed infection.

Sequencing

The PCR product from 4 samples with indeterminate RFLPs, of which 2 also had evidence of mixed infection by the type-specific PCR method, were sequenced (Figure 2). Cases 1, 2, and 3 showed significant sequence homology with genotype 1 (pairwise sequence similarity between positions −184 and −92 = 89.2, or 97.8%) and the sequence predicted binding with the genotype 1–specific primer. All these sequences had a restriction mapping pattern deviated from that of the predicted (Table III). The sequence of cases 1 and 2 did not predict binding with type 2– or type 3–specific primers although amplification products were available with the type 2– and the type 3–specific primers, yielding mixed infection of genotypes 1 and 2 for case 1 and

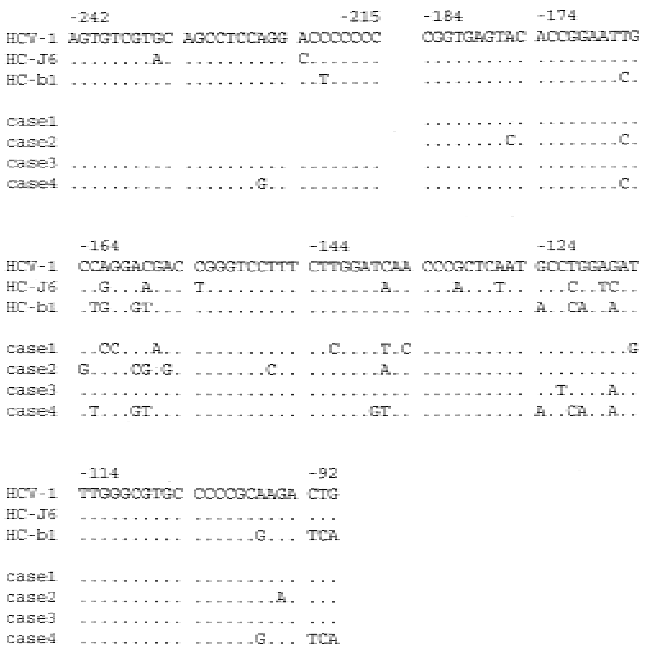

Fig. 2. Sequence alignment of 4 RFLP indeterminate cases and their comparison with the prototypes. HCV-1 = genotype 1a, HC-J6 = genotype 2a, HC-b1 = genotype 3a [Simmonds et al., 1993]. Sequence between positions –185 and –214 are identical with the prototype. Sequences for cases 1 and 2 between positions –242 and –215 could not be determined.

genotypes 1, 2, and 3 for case 2. The predominant band, however, came from genotype 1–specific primer and this was in accordance with the sequencing data. There were 2 changes seen in case 1, a change from A to C at position –161 and from G to C at position –162, which resulted in loss of both *MvaI* and *ScrfI* sites. Similarly, in case 2, a change from A to C at position –176 and from T to C at position –166 created sites for *HinfI* and another change from C to G at position –164 deleted the site for *MvaI* and *HinfI,* thereby altering the digestion patterns. Case 3 was almost identical in sequence (pairwise similarity between positions –184 and –91 = 97.8%) with that of HCV-1 [Simmonds et al., 1993]. A change from C to T at position –122 resulted in loss of *Mva1* site and changed the RFLP pattern. The type-specific PCR method correctly identified this pattern as type 1.

Case 4 showed significant sequence homology with genotype 3 (pairwise sequence similarity between positions –184 and –92 = 91.4%) and the sequence predicted binding with the genotype 3–specific primer. A sequence change in the position –225 from A to G rendered a loss of a *MvaI* site, although *ScrfI*/*HinfI* pattern remained as predicted. Type-specific PCR method had correctly identified this case as genotype 3.

DISCUSSION

A range of techniques have been described for genotyping hepatitis C virus, each with its own advantages and disadvantages. Direct sequencing of PCR product is impractical for routine use. The original RFLP method required radioisotope labelling [McOmish et al., 1994] which limits its general use although successful non-isotopic uses have been published [Majid et al., 1995; Davidson et al., 1995]. The original type-specific primers approach by Okamoto et al. [1992] amplified the core region, which is generally less conserved than the 5′ non-coding region. This method was criticised by some investigators as it produced a higher percentage of mixed infection, probably as a result of non-specific reactions with the subtype 1b primers [Lau et al., 1995; Kleter et al., 1995]; however, it was later modified to improve its functioning [Widell et al., 1994; Kleter et al., 1995]. The probe detection method and serotyping assays are both commercially available and appear to work well [Stuyver et al., 1993; Bhattacherjee et al., 1995; Smith et al., 1995]. These are, however, very costly assays. We therefore developed 2 simplified versions of the above, namely non-isotopic RFLP and type-specific primers. In addition, a subset of samples were examined using a commercial serotyping ELISA.

With the RFLP analysis, 7 samples (13%) had restriction digestion patterns that did not fall into any of the predicted patterns suggested by McOmish et al. [1994]. Further RFLP patterns were reported in the recent literature [Smith et al., 1995]. However, the 4 sequences from this study did not appear to match with any of the published patterns. Since restriction sites could be created or abolished with a single base mutation, it is possible that there are many more combinations of RFLP patterns within a genotype.

Both the RFLP method and serotyping ELISA used in this study are capable of identifying all the 6 major genotypes of HCV. The main genotypes in Western countries, however, are genotypes 1, 2, and 3 [Simmonds et al., 1993a; Dushieko et al., 1994; Takada et al., 1993; Lau et al., 1995] and this was borne out in the present study. Apart from 2 patients who had genotype 4 and genotype 6 as minor serotypes in mixed infections as detected by serotyping ELISA (Table II), none of the other patients had infection by genotypes other than 1, 2, and 3. The type-specific primers used in this study were based on available knowledge of the polymorphic sites in the 5′ NCR and were designed to have the 3′ end of the primer sitting on the specific polymorphic site. Variation in the 5′ NCR has been well studied and the genotypic predictive value of polymorphic sites confirmed [Smith et al., 1995]. It produced typeable results in all 64 samples. The subtype 1b primer in this study was designed based on the fact that the majority of type 1b sequences had a guanine base in position –99 [Smith et al., 1995]. The specificity of this primer was confirmed as only those samples typed as genotype 1 produced amplicons with the type 1b primer. However, these polymorphic sites on their own are by no means unique. Theoretically, from available sequences information in the GenBank, the genotype 1 specific primer used in this study could also amplify type 6, and the type 1b subtype specific primer could also amplify genotype 4 [Simmonds et al., 1993c]. In countries where genotype 4 and 6 also occurred, its use is therefore

TABLE III. Comparison of Results From the Different Methods in the 4 Cases Where Direct Sequencing Was Performed

Cases	Predicted RFLP patterns		Type specific PCR results	Serotype ELISA results	Sequence (% pairwise similarity to prototype)
	MvaI/HinfI	ScrfI/HinfI			
Case 1	112, 58	58, 41, 39, 32	1 > 2	not tested	Type 1 (92.5)
Case 2	59, 50, 48	59, 50, 48	1 > 2 > = 3	untypeable	Type 1 (89.2)
Case 3	92, 63	83, 48	1	1	Type 1 (97.8)
Case 4	99, 74	83, 41	3	not tested	Type 3 (91.4)

limited. However, sequence analysis suggests that it is also possible to design specific primers for genotype 4 and genotype 6 in the 5′ NCR [Smith et al., 1995], though this would increase the complexity of the assay. However, the assay in its present format will provide information about the presence or absence of the bad prognostic genotypes and may thus be of some use in clinical practice.

The most perplexing and controversial aspect is the phenomenon of mixed infection. The prevalence of mixed infection depended very much on the method used. Methods such as RFLP, which match genotypes with expected patterns, will not easily detect mixed infections. In a situation with true mixed infection, either only the predominant pattern prevails or an indeterminate pattern emerges as the mixed digestion pattern appear together. A study on HCV genotypes in haemophiliacs has shown that a combined RFLP and sequencing approach detected mixed infection in 7%, whereas a serotyping method identified 14% [Preston et al., 1995]. The type-specific primer approach using the core region has been associated in particular with detection of mixed infections [Lau et al., 1995]. More than 10% of patients with chronic liver disease had mixed genotype infection in one study from Taiwan using this typing method [Chen et al., 1994]. Here, use of the type-specific primer method for the 5′ NCR showed that 11/64 (17%) patients had mixed infection. The serotyping ELISA method identified 3/25 (12%) as mixed infection, 2 of which were the same samples identified by type-specific PCR as mixed infections. Interestingly, with these 2 patients, while 1 showed reasonable agreement between the 2 methods (Table II), the other had genotype 3 identified by type-specific PCR as the minor type and type 6 and type 2 were identified by serotyping ELISA as the minor types. Discrepancy is possible, however, as the serotyping assay detects both current and past infections, whereas the genotyping assay is for current infections only. The mixed infections were seen in haemophiliacs, intravenous drug users, and haemodialysis patients. These are the groups where multiple exposure is likely and mixed infections are possible.

Some studies have followed the courses of definite mixed infections, and although all combination of events could occur, at least in some patients there was a tendency to select for a particular type, usually the more pathogenic subtypes [Villa et al., 1995; Widell et al., 1995]. This was compatible with our findings that a predominant type was always identifiable in our patients. Alternative explanations are possible. For example, the presence of a wide range of quasispecies in some samples may lead to minor species with variations in the polymorphic sites and this could lead to amplification resulting in PCR products apparently specific for another genotype. If this is true, then the findings of these apparent mixed infections by type-specific PCR could be of some clinical interest as the presence of quasispecies has been linked with a bad prognosis [Moribe et al., 1995].

The sequence results from the 2 patients with evidence of mixed infection by the type-specific PCR method (cases 1 and 2) confirmed the validity of the predominant genotypes as there was significant sequence homology with the predominant genotype and it also predicted binding with the relevant primer. However, it did not resolve the problem of whether there were co-existing mixed infections with minor population of sequences or mispriming as a result of quasispecies. The real prevalence of mixed infection can be resolved only by cloning of the PCR products and undertaking multiple sequencing on different clones.

To conclude, the 2 simplified genotyping approaches appeared to produce valid and generally concordant results with the type-specific PCR method producing more typeable results than RFLP. With apparent mixed infections, while some cases probably had true mixed infection, some may be artefacts as a result of quasispecies.

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